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### **Remarks**

Applicants have amended claims 13, 18, 23, and 28 to more clearly define the claimed subject matter. Specifically, support for the added claim language can be found on page 99, lines 15-17 of the specification as filed. No new matter has been added.

Claims 1-52 are pending in the instant application.

#### **I. Rejection of Claims Under 35 U.S.C. § 112, Second Paragraph**

Claims 6, 12, 17, 22, 27, 32, 37, 42, 47 and 52 have been rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite because “proteins are not expressed.” *See* Paper No. 20031208, page 2.

Applicants respectfully disagree and traverse. Applicants assert that the term “expressing a protein” is scientifically proper and does not render the rejected claims indefinite. Biotechnology catalogs list numerous products related to protein expression, such as vectors that can be used to express proteins, cells used to express proteins, cell-free protein expression systems, *etc.* *See*, for example, Promega’s In Vitro Expression Guide, Chapter One, submitted herewith as Exhibit A. Applicants assert one of ordinary skill in the art would clearly recognize that proteins are indeed expressed and consequently, that the claims, as currently presented, are definite. In view of the above, Applicants respectfully request withdrawal and reconsideration of the rejection of the claims under 35 U.S.C. § 112, second paragraph.

#### **II. Rejection of Claims Under 35 U.S.C. § 112, First Paragraph**

The Examiner has rejected claims 13-32 under 35 U.S.C. § 112, first paragraph. *See* Paper No. 20031208, page 2. In particular, the Examiner asserts:

The specification does not contain any disclosure of the function of all proteins that are 90% identical or 95% identical to SEQ ID NO:310 or some portion thereof or the polypeptide encoded by the HEMA80 cDNA contained in ATCC Deposit No. 97975 or some portion thereof. The genus of proteins that comprise these above protein molecules is a large variable genus with the potentiality of possessing many different functions. Therefore, many functionally unrelated proteins are encompassed with the scope of these claims, including partial protein sequences. The specification discloses only a single species of the claimed genus, which is insufficient to put one of skill in the art in possession of the attributes and features

of all species within the claimed genus. Therefore, one skilled in the art cannot reasonably conclude that the applicant had possession of the claimed invention at the time the instant application was filed.

See Paper No. 20031208, page 3, lines 6-16.

Applicants respectfully disagree and submit that the test for the written description requirement is whether one skilled in the art could reasonably conclude that the inventor has possession of the claimed invention in the specification as filed. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 U.S.P.Q.2d 1111, 1116 (Fed. Cir. 1991); M.P.E.P. § 2163.02.

The Federal Circuit recently re-emphasized the well-settled principle of law that “[t]he written description requirement does not require the applicant ‘to describe exactly the subject matter claimed, [instead] the description must clearly allow persons of ordinary skill in the art to recognize that [they] invented what is claimed,’” *Union Oil Co. v. Atlantic Richfield Co.*, 208 F.3d 989, 54 U.S.P.Q.2d 1227 (Fed. Cir. 2000), hereinafter referred to as “*Unocal*.” While the applicant must “blaze marks on trees,” rather than “simply [provide] the public with a forest of trees,” an Applicant is not required to explicitly describe each of the trees in the forest. See *Unocal*, 208 F.3d at 1000. See also M.P.E.P. § 2163.02 (“The subject matter of the claim need not be described literally (*i.e.*, using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement.”). The Court emphasized the importance of what the person of ordinary skill in the art would understand from reading the specification, rather than whether the specific embodiments had been explicitly described or exemplified. As the court noted, “the issue is whether one of skill in the art could derive the claimed ranges from the patent’s disclosure.” *Unocal*, 208 F.3d at 1001 (emphasis added).

Applicants recognize that the Examiner is in part relying on language regarding a “lack of description of representative species” of a claimed genus set forth in *Regents of the University of California v. Eli Lilly & Co.*, (119 F.3d 1559, 1569, 43 U.S.P.Q.2d 1398, 1406 (Fed. Cir. 1997)) (hereinafter “*Eli Lilly*”) and incorporated into the Guidelines for Examination of Patent Applications Under the 35 U.S.C. § 112, ¶ 1 “Written Description” Requirement (“Guidelines”), when reciting the procedures followed in analyzing whether the description requirement for each of the claims at issue is satisfied. The central issue in *Eli Lilly* involved claims to all mammalian cDNAs encoding insulin, which were

supported in the specification only by the nucleotide sequence for the rat insulin gene. The Federal Circuit found the claims to human insulin lacked written description because the claims defined only a result or function. The court held that a result or function would satisfy the written description requirement *only if* correlated to a description of structural features of the claimed invention. According to the court, a sufficient written description must allow the skilled artisan to "visualize or recognize the identity of the members of the genus." *Id.*

In addition, the court held in *Eli Lilly* that a description of a genus of cDNAs may be achieved by reciting a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus *or* by reciting structural features common to a substantial portion of the members of the genus. *Eli Lilly*, 119 F.3d 1559, 1569 (Fed. Cir. 1997). Therefore, it logically follows that claims to polypeptides encoded by cDNAs may also be satisfied by providing sequences of a representative number of polypeptides which fall within the scope of the genus *or* by providing a recitation of structural features common to a substantial portion of the members of the genus.

Preliminarily, Applicants point out that claims 13, 18, 23, and 28 have been amended to recite a biological function to encompass species possessing the same functional activity as SEQ ID NO:310 or the polypeptide encoded by the HEMA80 cDNA contained in ATCC Deposit No. 97975. As discussed above, support for the amendment may be found, for example, on page 99, lines 15-17, of the specification. Since Applicants have disclosed species representative of the entire genus, one of ordinary skill in the art would reasonably conclude that Applicants were in possession of the claimed genus. Applicants assert that these claims, as amended, fully meet the written description requirement since one of ordinary skill in the art can readily envision all species encompassed by the claims.

Applicants assert that, in the instant case, the second test set forth in *Eli Lilly* has been satisfied because Applicants' description of the reference polypeptide sequence, SEQ ID NO:310, provides one skilled in the art with the necessary structural features common to a substantial portion of the members of the genus. Applicants further point out that the recitation of the structural features of the reference protein is a recitation of the structural features common to the members of the claimed genus because the proteins included within the claimed genus will have at least 90% (or at least 95%) of the amino acids of

their amino acid sequence primary structure in common to the reference polypeptide of SEQ ID NO:310. Indeed, nothing more than a basic knowledge of the genetic code and what is described in the specification (*i.e.*, pages 366, line 11 to page 371, line 36 of the specification) would be required for the skilled artisan to identify every single one of the polypeptides that are 90% or 95% identical to the amino acid sequence of SEQ ID NO:310 having cell proliferative activity. Clearly, such knowledge is well within what is expected of the skilled artisan. Therefore, in accord with *Eli Lilly*, the specification clearly conveys that Applicants were in possession of the claimed invention on the priority date of the instant application.

In view of the arguments above and amendments made herein, Applicants submit that the pending claims fully meet the written description requirements of 35 U.S.C. § 112, first paragraph, and respectfully request that the Examiner's rejection of claims 13-32 under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

### **III. Claim Rejections Under 35 U.S.C. §§ 101/112, First Paragraph**

The Examiner has rejected claims 1-52 under 35 U.S.C. § 101 because the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility. In particular, the Examiner asserts on page 4, lines 12-18 of the Office Action (Paper No. 20031208):

The only disclosure of this invention is on pp 98-99 and consists of potential uses for the protein. The gene seems to be expressed primarily in fetal liver and fetal spleen and many conclusions of utilities are drawn from this fact. However, the location of expression of the gene is not enough to establish a specific and substantial utility. For example, page 99, lines 21-22 state that the protein may be involved in apoptosis or tissue differentiation and could be useful in cancer therapy. While this may be true, it does not constitute a specific utility since there are so many types of cancer.

Applicants respectfully disagree and traverse. In order to find that an asserted utility is neither specific nor substantial, the burden is on the Examiner to make a *prima facie* case showing that it is more likely than not that a person of ordinary skill in the art would not consider any utility asserted by the Applicant to be specific or substantial. See M.P.E.P. § 2107.02(IV); Utility Examination Guidelines, 66 FR 1092, January 5, 2001 at

1098, col. 3 (emphasis added). In the instant case, Applicants respectfully assert that the Examiner has not met the burden required to establish a rejection under 35 U.S.C. § 101, as one of ordinary skill in the art would unlikely question the truth of Applicants' asserted utility. In support of the 35 U.S.C. § 101 rejection, the Examiner has simply noted that the location of a gene does not render a specific and substantial utility, and that the asserted utility is not specific due to the numerous types of cancers. Applicants respectfully submit that the burden required to establish a rejection under 35 U.S.C. § 101 has not been met.

Applicants note that specific, substantial and credible utilities were asserted in the specification. *See*, for example, page 99, lines 5-17. Indeed, the Federal Circuit clearly held that "[w]hen a properly claimed invention meets at least one stated objective, utility under 35 U.S.C. § 101 is clearly shown." *Raytheon v. Roper*, 724 F.2d 951, 958 (Fed. Cir. 1983). Applicants assert that the claimed polypeptide is useful for the diagnosis and treatment of conditions which include, but are not limited to, specific hematopoietic disorders, such as anemia, pancytopenia, leukopenia, thrombocytopenia or leukemia, where a shortage in the number of hematopoietic cells contribute to the diseases and where bone marrow transplantation and/or reconstitution can be used to treat such diseases. *See* page 99, lines 7-13 of the specification.

The Examiner alleges that Applicants' asserted utilities are not specific for the reasons stated in Paper No. 20031208, page 4, lines 14-18. Applicants respectfully disagree and traverse. The test for specificity is whether an asserted utility is specific to the subject matter claimed, in contrast to a utility that would be applicable to the broad class of the invention. *See* M.P.E.P. § 2107.01 on page 2100-32. All that is required of Applicants is that there be a reasonable correlation between the biological activity and the asserted utility. *See Nelson v. Bowler*, 626 F.2d 853, 857 (C.C.P.A. 1980) (emphasis added). The disclosed utilities for the claimed invention discussed above are specific, in that not every protein is useful for the diagnosis and/or treatment of the above-mentioned hematopoietic diseases. *See* page 99, lines 8-11 of the specification as filed. Furthermore, the expression of the gene in fetal liver and fetal spleen would be significant to one of skill in the hematopoietic art because it was well-known prior to the filing of the present invention that hematopoietic stem cells are found in the liver and spleen during fetal development. *See* Liu et al., page 286, first column; Genomics (2000) 65:283-292; previously submitted as Reference AD of Form PTO/SB/08A. Consequently, it is

reasonable to correlate the expression of the claimed invention to the utilities involving the specific hematopoietic disorders discussed above, thereby satisfying a specific utility.

The Examiner further alleges that the claimed invention is not supported by a substantial utility. As discussed above, Applicants assert that based on what is disclosed in the specification about the claimed invention, coupled with what was well-known in the art at the time the invention was filed, it is reasonable to assert that the claimed invention is useful in the diagnosis and/or treatment of certain hematopoietic disorders. The M.P.E.P. states, "any reasonable use that an applicant has identified for the invention that can be viewed as providing a public benefit should be accepted as sufficient, at least with regard to defining a 'substantial' utility." *See* M.P.E.P. § 2107.01(I). Applicants thus assert the claimed invention is supported by a substantial or "real world" utility.

Finally, while the Examiner did not raise credibility of the asserted utilities, Applicants respectfully submit that the specification also asserts credible utilities. The burden is on the Examiner to establish why it is more likely than not that one of ordinary skill in the art would doubt (*i.e.*, "question") the truth of the statement of utility. M.P.E.P. § 2107 at 2100-30 and 2100-40. The Examiner must provide evidence sufficient to show that the statement of asserted utility would be considered "false" by a person of ordinary skill in the art. *Id.* The Examiner must also present countervailing facts and reasoning sufficient to establish that a person of ordinary skill would not believe the Applicants' assertion of utility. *See Id.*; *see also, In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995).

Applicants assert that this gene product may be useful in the expansion of stem cells and committed progenitors of various blood lineages in order to diagnose and/or treat the above-discussed hematopoietic disorders. *See* page 99, lines 15-16 of the specification as filed. Indeed, third-party data supports this asserted utility. Liu *et al.* (Reference AD) identifies a secreted protein 100% identical to the claimed HEMA80 polypeptide, (referred to as C17 in their manuscript; see alignment submitted herewith as Exhibit B) selectively expressed on a subset of hematopoietic stem cells (*i.e.*, CD34+ cells). Liu *et al.* disclose that C17 was predominantly identified to be expressed in the fetal liver and fetal spleen, and these tissues are the primary hematopoietic organ during fetal development before bone marrow hematopoiesis is established. *See* Liu *et al.* (2000) page 286, first column. Therefore, C17 is likely involved in cell proliferative activities for hematopoietic stem cells. *See Id.* at page 291, right column. Combined with the data from

Liu *et al.*, one of ordinary skill in the art would, more likely than not, conclude that Applicants' asserted utility is specific, substantial and credible.

Additional support can also be found in two International Applications published after the filing date of the instant application. WO 00/56889 (previously submitted as Reference AA of Form PTO/SB/08A) and WO 02/00690 (currently submitted as reference AZ in a Supplemental IDS) demonstrate that HEMA<sup>80</sup> (referred to as PRO4425 in both applications; see alignment submitted herewith as Exhibit C) can induce proliferation of kidney mesangial and umbilical vein endothelial cells, respectively. *See* WO 00/56889 example 41 and WO 02/00690, example 21. Applicants note that it was well-known in the art that kidney mesangial cells are often used to test for cell proliferative activity as these cells are known to be responsive to proliferative cytokines. *See* Makino *et al.*, page 1140, right hand column; Makino *et al.* (2000) Nephrol Dial Transplant 15:1140-1145; submitted herewith as Exhibit D. It was also well-known that human umbilical vein endothelial cells are similarly used to test for possible growth factor functions, as discussed in example 21 of Reference AZ. This corroborates Applicants' asserted utility that the claimed invention may be commercially useful in cell differentiation and/or proliferation, particularly of hematopoietic stem cells. *See* page 99, lines 15-17 of the specification.

Applicants assert that scientific evidence may be used to corroborate Applicants' asserted utility. Legal precedent for the use of post-filing date references in this manner can be found in *In re Brana*, where the courts stated:

The Kluge declaration, though dated after applicants' filing date, can be used to substantiate any doubts as to the asserted utility since this pertains to the accuracy of a statement already in the specification. *In re Marzocchi*, 439 F.2d at 224 n.4, 169 U.S.P.Q. (BNA) at 370 n.4.

*See In re Brana*, 51 F.3d 1560 at 1567 n.19, 34 U.S.P.Q.2D (BNA) 1436 (March 30, 1995). Therefore, Applicants have established a credible utility for the claimed invention.

In view of the above arguments, Applicants have provided evidence and reasoning which supports the Applicants' assertion of a patentable utility. The utilities asserted in the specification for Secreted Protein HEMA<sup>80</sup> are specific, substantial and credible. Accordingly, Applicants respectfully submit that the rejection of claims 1-52 under 35 U.S.C. § 101 has been obviated. Therefore, Applicants respectfully request that the rejection be reconsidered and withdrawn.



For the reasons discussed above in response to the rejection under 35 U.S.C. § 101, the claimed invention is supported by a specific, substantial and credible asserted utility. The Examiner “should not impose a 35 U.S.C. § 112, first paragraph, rejection grounded on a ‘lack of utility’ basis unless a 35 U.S.C. §101 rejection is proper.” M.P.E.P. § 2107 (IV) at 2100-36. Therefore, because the claimed invention complies with the utility requirement of 35 U.S.C. § 101, the rejections under 35 U.S.C. § 112, first paragraph, based on the alleged lack of utility of the claimed invention, should be withdrawn. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

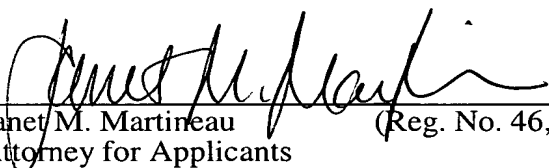
### *Conclusion*

Applicants respectfully request the amendments and remarks of the present response be entered and made of record in the present application. In view of the foregoing amendment and remarks, Applicants believe they have fully addressed the Examiner's concerns and that this application is now in condition for allowance. An early notice to that effect is urged. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the allowance of this application.

Applicants believe that there are no fees due in connection with the filing of this paper. However, should a fee be due, please charge the fees to our Deposit Account No. 08-3425. If a fee is required for an extension of time under 37 C.F.R. § 1.136, such an extension is requested and the appropriate fee should also be charged to our Deposit Account.

Respectfully submitted,

Date: March 11, 2004

  
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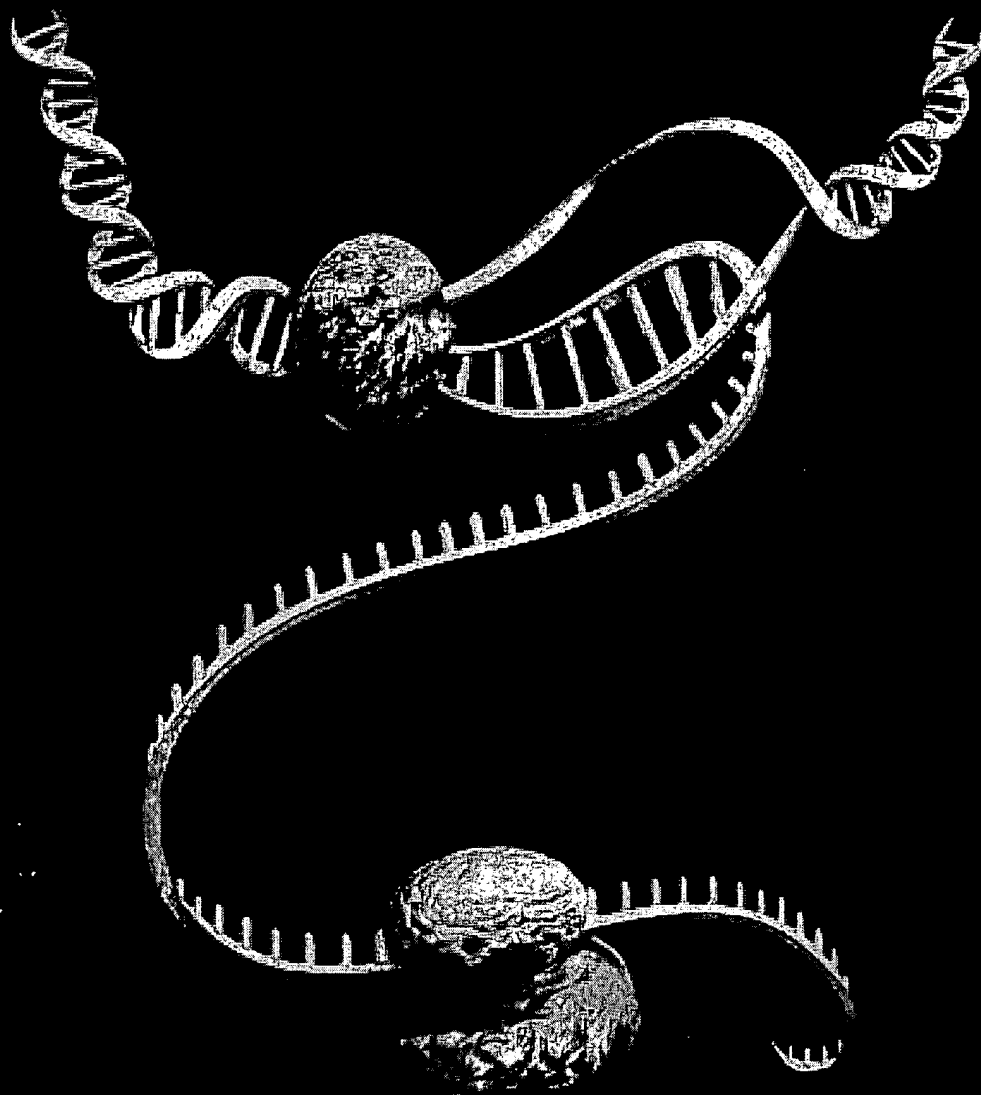
# CHAPTER

# ONE

## OVERVIEW

*"There is a single  
light of science,  
and to brighten  
it anywhere is  
to brighten it  
everywhere."*

*- Isaac Asimov -*



# PROMEGA IN VITRO RESOURCE

## Chapter One: Overview

Contents	Page
Introduction .....	3
Applications:	
<i>Genetic Verification and Detection</i> .....	4
<i>Functional Analyses</i> .....	4
<i>Molecular Interaction Detection</i> .....	4
<i>Molecular Structure and Localization Analyses</i> .....	5
<i>Molecular Diagnostics</i> .....	5
<i>High-Throughput Screening</i> .....	6
<i>Functional Genomics</i> .....	6
<i>Preparative Synthesis</i> .....	7
<i>Guide Organization</i> .....	7

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**About the Image:** In this illustration of transcription and translation, the DNA double helix separates at the RNA polymerase molecule with an mRNA strand extending downward from the RNA polymerase. Towards the 5'-end of the mRNA the two subunits of a ribosome assemble to translate the RNA message into a polypeptide chain, which will fold into a protein molecule. Throughout this In Vitro Expression Guide various and recent applications of translation and protein expression are discussed.



## Introduction

The use of cell-free systems for the in vitro expression of proteins is a rapidly growing area, with applications in basic research, molecular diagnostics and high-throughput screening. In vitro expression encompasses two general strategies. The first is to use isolated RNA synthesized in vivo or in vitro as a template for the translation reaction (e.g., using Promega's Rabbit Reticulocyte Lysate<sup>(a,b,c)</sup> (Cat.# L4151) or Wheat Germ Extract (Cat.# L4380) Systems). The second is to use a coupled transcription/translation system in which DNA is used as a template (e.g., Promega's TNT<sup>®</sup> <sup>(a,b,c,d,e)</sup> and *E. coli* S30 Extract<sup>(a,b)</sup> Systems). This DNA may be either a gene cloned into a plasmid vector (cDNA) or a PCR<sup>(f)</sup>-generated template.

In vitro expression is a rapidly growing and constantly evolving field. This guide is intended to provide a general overview of the technology as presented in recent scientific publications.

We wish to thank Wiley-Liss, a subsidiary of John Wiley & Sons, Inc., for their permission to reproduce much of the material in the chapter by Jagus, R. and Beckler, G. (1998) Overview of eukaryotic in vitro translation and expression systems, *Current Protocols in Cell Biology*, 11.1.1–11.1.13, in this guide.

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## Applications

### Genetic Verification and Detection

**Open Reading Frame (ORF) Expression.** Probably still the most common use of in vitro expression is simply to decode a nucleic acid to determine if a gene or ORF is present. In DNA, an ORF consists of an initiation codon (usually ATG, but can also be GTG), followed by a sequence of nucleotides coding for amino acids and ending with a termination codon (TAA, TAG or TGA).

For example, a prokaryotic DNA (or eukaryotic cDNA) clone can be analyzed for the presence of ORFs using an in vitro expression system. Alternatively, in vitro expression can be used to verify an ORF predicted by DNA sequencing. In each case, the resulting proteins are characterized, and the protein size and structure are correlated to the size and sequence of the gene.

Specific uses include analysis of viral RNA genomes in which eukaryotic translation systems are used to determine the number and function of the viral genes (1), and study of differential protein expression using total cellular mRNA or polysomes from different tissues.

**Cloned cDNA Expression.** Cloned cDNAs positioned behind a phage promoter (e.g., T7, T3 or SP6) are commonly used for generating gene-specific mRNA used to program translation reactions (2). Run-off 5'-capped or uncapped mRNA can be produced in vitro and added to translation extracts. Alternatively, these DNA constructs can be used directly in eukaryotic coupled transcription/translation reactions.

### Functional Analyses

**Enzymatic Activity Analysis.** Many proteins expressed using in vitro systems are correctly folded and processed and display normal in vivo enzymatic activity. If the extract system itself lacks (or has low levels of) the enzymatic activity of the expressed protein, the resulting translation reaction can be assayed directly without protein purification. Another advantage of in vitro expression is the ability to add exogenous factors to study enzymatic activity, potentially eliminating the need for transfection studies.

In one example, expression of adenylate cyclase (ACIV; 110kDa) in a TnT® System produced a protein with the same specific enzymatic activity as ACIV produced from a baculovirus expression system (3). In another example, aromatase produced in a TnT® System reaction supplemented with canine pancreatic microsomes and recombinant cytochrome P<sub>450</sub> reductase resulted in an active enzyme (4).

**Mutation Analysis.** Upon cloning a gene, a number of studies are undertaken to discern the

function of a gene product. A first step is often the introduction of a mutation into the gene to examine the effect on the expressed protein. Methods of mutagenesis include: i) serial deletion mutation analysis by progressive truncation of the 5'- or 3'-ends of the gene or by using *Bal* 31 digestion from an internal single-cut restriction site; and ii) site-directed point mutation analysis (e.g., using Promega's GeneEditor™ System<sup>(9)</sup>). Both methods can be used to identify functionally active domains or residues.

**Post-Translational Modification Analysis.** Post-translational modification of the protein, such as proteolytic cleavage or the addition of sugars, lipids, phosphate or adenylyl groups, is often required for functional activity. Each in vitro expression system has its own endogenous post-translational modification activities. For example, various phosphorylation, adenylation, myristoylation, farnesylation, isoprenylation and proteolytic activities have been observed using rabbit reticulocyte lysate. Addition of microsomal membranes allows the study of glycosylation, methylation and removal of signal sequences.

Because not all the differences between the various in vitro expression systems are known, it may be desirable to try both reticulocyte lysate and wheat germ extract (and in some cases, *E. coli* S30 extract) to determine which system can produce a functional gene product with the "correct" post-translational modifications. In addition, cellular extracts or different microsomal membrane sources (e.g., *Xenopus* egg extracts) can be added to provide additional modifying activities.

### Molecular Interaction Detection

**Protein-Protein.** Specific protein-protein interactions can be detected using in vitro expression methods. These interactions may include specific binding (such as antibody-antigen and ligand-receptor binding), macromolecular assembly, and formation of functional transcription complexes. In a common application, one protein partner is expressed in large amounts and purified from *E. coli* as a fusion protein. The other partner is expressed in an in vitro expression system as a labeled protein and used as a probe for detection of the interaction. Often, this technique is used to verify results from yeast two-hybrid experiments. A variety of biochemical analysis methods may be used to characterize the expressed proteins.

**Protein-DNA and Protein-RNA.** Putative DNA binding proteins, such as transcription factors, can be analyzed for their ability to bind to specific sequences on radiolabeled oligonucleotides. The binding is detected by an electrophoretic mobility shift assay (EMSA) in

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which greater retardation of the protein-DNA complex is observed when compared to unbound DNA. Usually the labeled DNA is added directly to the in vitro expression reaction. Researchers studying transcription factors such as NF- $\kappa$ B often use wheat germ extracts, as they do not contain endogenous mammalian transcription factors. A method has been reported to remove endogenous DNA-binding proteins from the reticulocyte system prior to the translation reaction (5).

**DNA-RNA and RNA-RNA.** Antisense DNA oligonucleotides can be useful for inhibiting expression at both the transcription and translation level. TNT® Systems have been used to rapidly screen oligonucleotides for those that best arrest translation (6,7).

## **Molecular Structure and Localization Analyses**

**Characterization of Membrane Association.** In vitro expression systems have been successfully used to express integral membrane proteins. For example, expression of G-protein-coupled receptors in TNT® Systems supplemented with canine microsomal membranes results in the correct folding and insertion of transmembrane domains using the expressed signal anchor and stop transfer sequences (8,9)

**Non-Natural Amino Acid Incorporation.** Using technology originally developed in 1976, Johnson revolutionized in vitro translation by demonstrating that non-natural amino acids could be inserted into polypeptides using epsilon-modified, lysine-charged tRNAs (10). Further extension of this technology led to the incorporation of photoactivatable crosslinking or fluorescent groups into polypeptides, followed by monitoring of the molecular environment as the labeled peptides pass through the ribosome and enter the endoplasmic reticulum pore (11,12). Site-directed incorporation of a photoactivatable crosslinker through a non-natural amino acid incorporation was used to capture protein interacting with different portions of a protein of interest (13). Other groups have developed site-specific methods that utilize an amber suppressor tRNA charged with any number of non-natural amino acids, including fluorescent, spin-label and isotopic groups (14).

For a review of tRNA-mediated protein engineering (TRAMPE) see reference 15.

**Protein Folding and Chaperonin Interactions.** In vitro expression is increasingly being used to understand the nature of sequential chaperonin interactions required for protein folding and localization. Researchers in this field have combined the advantages of in vitro expression with the power of instantaneous reporter gene product assays. The folding of polypeptides emerging from ribosomes has been analyzed

using firefly luciferase as a model protein (16,17).

**Real-Time Translation/Folding Assays.** A novel approach has been developed using a wheat germ system in which the components for the luciferase enzymatic assay have been added directly to the translation reaction and monitored continuously in real time. Luciferase was shown to be fully folded and enzymatically active immediately upon release from the ribosome (18). However, no luciferase activity was observed while full-length luciferase remained attached to the ribosome as a peptidyl-tRNA, probably because the C-terminal portion of the enzyme is masked by the ribosome or ribosome-associated proteins. The investigators demonstrated that the ribosome-bound enzyme acquires enzymatic activity when its C-terminus is extended by at least 26 additional amino acid residues (19). The results demonstrate that the acquisition of the final native conformation by a nascent protein can occur as the protein is being synthesized and that folding does not require release of the protein from the ribosome.

**Macromolecular Assembly.** It is possible to express numerous gene products in one coupled transcription/translation reaction to form functional transcription factor complexes (20) or viral particles (21) that are identical to those formed in the host.

**Molecular Structure Analysis.** Understanding the function of integral membrane proteins is currently limited by the difficulty of producing crystals for use in X-ray diffraction studies. A method has been developed for probing conformational changes in membrane proteins using Fourier transform infrared-difference (FTIR) spectroscopy. In this method, natively folded polypeptides are expressed in vitro with a site-specific insertion of a single isotopic label through amber suppression (22). This method does not disrupt the protein structure as did earlier site-directed mutagenesis methods and should be applicable to a wide range of other proteins, including those involved in enzyme catalysis, ion transport and signal transduction.

## **Molecular Diagnostics**

**Protein Truncation Test.** A growing application of coupled transcription/translation systems has been for diagnosis of genetic diseases, a DNA technology-dominated field. The protein truncation test (PTT), sometimes referred to as in vitro synthesized protein truncation (IVSP) assay, was first reported in 1993 as a rapid method for detecting translation-terminating mutations in the large gene responsible for Duchenne Muscular Dystrophy (23) and the Adenomatous Polyposis Coli (APC) gene responsible for a type of hereditary colon cancer (24). In these and other

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diseases, such as hereditary breast cancer (24), 70–95% of the mutations that cause disease result in a truncated gene product.

The PTT involves first purifying genomic DNA or mRNA from the patient's blood or tissue. This is followed by either RT-PCR<sup>®</sup> or PCR<sup>®</sup> with the concurrent incorporation of a T7 promoter and optimal translation initiation sequence surrounding the desired start codon (25). Often when the source of mRNA is limiting, a second nested PCR amplification is required. Large exons are amplified from genomic DNA while smaller exons are amplified together from mRNA and the gene is segmented into overlapping amplified fragments. The amplified DNA is added directly to a coupled transcription/translation reaction and translation terminating mutations are detected as faster migrating bands after SDS-PAGE analysis. PTT has the advantage of enabling scans of large (2–3kb) DNA/RNA segments quickly. In addition, PTT detects only disease-causing mutations. This avoids the fruitless evaluation of polymorphisms. The recent introduction of the TNT<sup>®</sup> T7 Quick for PCR DNA System<sup>(c.d.s.g.)</sup> facilitates PTT analysis.

### High-Throughput Screening (26)

**Screening for Viral-Specific Translation Inhibitory Compounds.** Viruses contain a number of different genetic elements used for promoting viral expression at the expense of host mRNA translation. Several groups are currently developing screens using in vitro expression of gene constructs containing a viral element such as the 5'-UTR that can harbor an IRES (Internal Ribosome Entry Site) followed by a firefly or *Renilla* luciferase gene (27). Chemical or antibiotic libraries can be screened for specific translation-inhibiting effects. The viral element can be placed between the firefly and *Renilla* luciferase genes with translation of the first gene relying on normal cap-dependent initiation. Use of the two luciferase genes allows normalization of the second reporter behind the viral element. The efficacy of compounds can be assessed rapidly (<30 seconds) by assaying light output from both the reporter and the control luciferase (Figure 1, Chapter 5).

As a variation on this theme, RiboGene, Inc., has reported developing a high-throughput system for screening several hundred thousand compounds for the ability to diminish or block the required ribosomal frameshifting used during translation of the HIV gag-pol mRNA. This screen utilizes a reporter gene in which luciferase (and light) is produced only when the frameshift occurs.

**Screening for Chaperonin-Inhibiting Drugs.** The in vitro luciferase folding/chaperonin assay

described earlier has been extended to understanding the role of heat shock factors, such as Hsp90. It is now understood that disruption of the folding pathways can result in proteolytic degradation. Several groups are currently using this information to ascertain the pharmacological activities of benzoquinone ansamycins, such as geldanamycin (28,29). These potentially medically important compounds were first identified as interesting because of their ability to inhibit tyrosine kinase activity. This ability appears to be due to their interaction with Hsp90, which prevents the correct folding of tyrosine kinases and is followed by their proteolytic degradation. Other potentially important drugs affecting protein folding through inhibition of chaperonin function could be identified using this approach.

**Identification of Novel Orphan Receptors.** The binding of ligands to in vitro synthesized receptors can be an important aspect of identifying new receptors. For example, in a search for novel "orphan" nuclear receptors and ligands, a novel estrogen receptor was cloned and characterized (30). Saturation ligand-binding and ligand-competition assays of the in vitro expressed clone allowed this novel receptor to be distinguished from a previously cloned receptor.

### Functional Genomics

**In Vitro Expression Cloning (IVEC).** In this procedure, an oligo(dT)-primed cDNA library is constructed in a high copy expression plasmid containing a T3, T7 or SP6 promoter. The plasmid library is then transformed into *E. coli*, and approximately 10<sup>5</sup> independent transformants are plated on selective media. The bacterial colonies are grown to a specific size (e.g., 1mm in diameter), collected and pooled (50–100 clones per pool). Purified plasmid DNA from these pools is directly added to a small-scale (e.g., 10µl) coupled transcription/translation reaction, where it is used as a template in the presence of [<sup>35</sup>S]methionine (31). Depending upon the number of the full-length cDNA clones in the library, approximately 30–50 proteins can be produced in a single reaction. Proteins can be assayed for any number of activities, including phosphorylation, proteolysis or cleavage. Positive pools are subdivided until the single cDNA that encodes the protein of interest is isolated.

**Ribosomal Display for Cell-Free Protein Evolution.** In this procedure, a cell-free system is used to transcribe a DNA library, translate the mRNA pools and, using a variety of techniques, the proteins and the encoding mRNAs are retained, still attached to the ribosomes. The protein-mRNA-ribosome complexes are screened for binding to a target, and the retained mRNA is

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amplified using RT-PCR<sup>®</sup> with the resulting DNA used for another round of selection. Initially, a prokaryotic *E. coli* coupled transcription/translation system was used to generate large libraries of peptides for receptor ligand screening (32). Later improvements allowed folding of whole proteins into their native structure while still attached to the ribosome (33). The first eukaryotic application used a coupled rabbit reticulocyte system to study antibody-ribosome-mRNA (ARM) complexes, allowing for rapid selection and monitoring of antibody combining site evolution (34).

A cell-free system has been developed for performing evolution studies in which RNA amplification and the coupled reaction can be performed simultaneously at a given temperature (35). After unsuccessful attempts using wheat germ extracts and coupled *E. coli* systems, investigators were able to combine the reactions using a rabbit reticulocyte coupled system. By exerting selective pressure on functional protein products necessary for RNA amplification, this system can be used for performing laboratory "evolution."

## Preparative Synthesis

### Large-Scale Protein Expression and Purification.

Cell-free expression systems are often preferred over in vivo or native systems, because they can be used for the expression of toxic, proteolytically sensitive or unstable proteins. In addition, in vitro systems provide the ability to incorporate non-natural amino acids containing photoactivatable, fluorescent or biotin residues. Typically, in vitro systems produce nanogram amounts of proteins per 50µl reaction; however, preparative scale methods have been developed recently that may yield milligram quantities per milliliter of reaction mixture.

## Guide Organization

This chapter provides a general overview of many of the major applications of in vitro expression systems. The remainder of the guide focuses on several of these applications, seeking to provide more detailed information on commonly used methodologies for in vitro expression technology.

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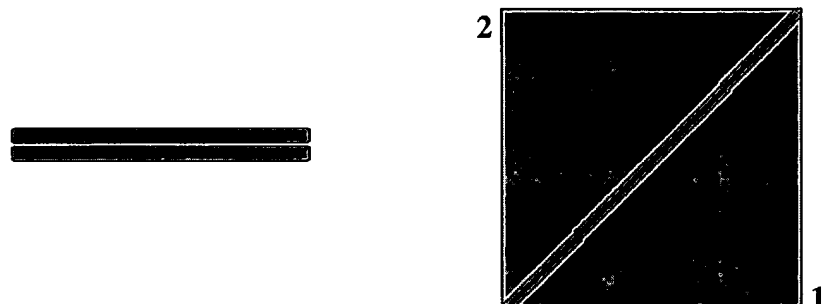
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# Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

## BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.6 [Apr-09-2003]

Matrix: **BLOSUM62** ☒ gap open: **11** gap extension: **1**x\_dropoff: **50** expect: **10.000** wordsize: **3** Filter ☐ **Align****Sequence 1** lcl|AAW88595 Secreted protein encoded by gene 62 clone HEMAE80 **Length** 136 (1 .. 136)**Sequence 2** gi 8922108 cytokine-like protein C17 [Homo sapiens] **Length** 136 (1 .. 136)

NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 283 bits (724), Expect = 6e-76

Identities = 136/136 (100%), Positives = 136/136 (100%)

```
Query: 1   MRTPGPLPVLLLLLAGAPAAARPTPPTCYSRMRALSQEITRDFNLLQVSEPSEPCVRYLPR 60
          MRTPGPLPVLLLLLAGAPAAARPTPPTCYSRMRALSQEITRDFNLLQVSEPSEPCVRYLPR
Sbjct: 1   MRTPGPLPVLLLLLAGAPAAARPTPPTCYSRMRALSQEITRDFNLLQVSEPSEPCVRYLPR 60

Query: 61  LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA 120
          LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA
Sbjct: 61  LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA 120

Query: 121 LEYPIPVTTVLPDRQR 136
          LEYPIPVTTVLPDRQR
Sbjct: 121 LEYPIPVTTVLPDRQR 136
```

CPU time: 0.02 user secs. 0.00 sys. secs 0.02 total secs.

Lambda	K	H
0.326	0.142	0.448

Gapped Lambda	K	H
0.267	0.0410	0.140

Matrix: BLOSUM62

Gap Penalties: Existence: 11, Extension: 1

Number of Hits to DB: 322

Number of Sequences: 0

Number of extensions: 11

Number of successful extensions: 1

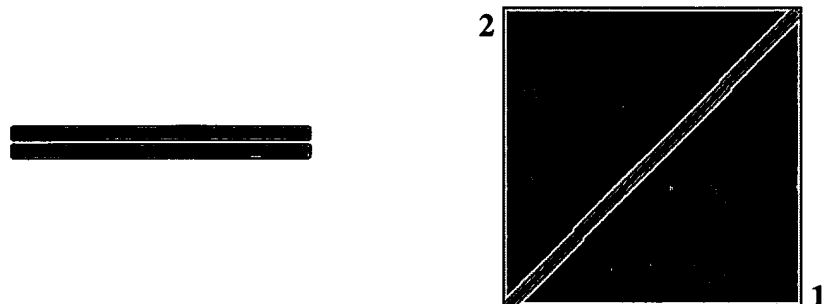
Number of sequences better than 10.0: 1  
Number of HSP's better than 10.0 without gapping: 1  
Number of HSP's successfully gapped in prelim test: 0  
Number of HSP's that attempted gapping in prelim test: 0  
Number of HSP's gapped (non-prelim): 1  
length of query: 136  
length of database: 547,437,766  
effective HSP length: 112  
effective length of query: 24  
effective length of database: 547,437,654  
effective search space: 13138503696  
effective search space used: 13138503696  
T: 9  
A: 40  
X1: 15 ( 7.0 bits)  
X2: 129 (49.7 bits)  
X3: 129 (49.7 bits)  
S1: 40 (21.6 bits)  
S2: 67 (30.4 bits)



# Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

## BLAST 2 SEQUENCES RESULTS VERSION BLASTP 2.2.6 [Apr-09-2003]

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NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

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Identities = 136/136 (100%), Positives = 136/136 (100%)

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          MRTPGPLPVLLLLLAGAPAAARPTPPTCYSRMRALSQEITRDFNLLQVSEPSEPCVRYLPR
Sbjct: 1  MRTPGPLPVLLLLLAGAPAAARPTPPTCYSRMRALSQEITRDFNLLQVSEPSEPCVRYLPR 60

Query: 61 LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA 120
          LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA
Sbjct: 61 LYLDIHNYCVLDKLRDFVASPPCWKVAQVDSLKDKARKLYTIMNSFCRRDLVFLDDCNA 120

Query: 121 LEYPIPVTTVLPDRQR 136
          LEYPIPVTTVLPDRQR
Sbjct: 121 LEYPIPVTTVLPDRQR 136
```

CPU time: 0.01 user secs. 0.00 sys. secs 0.01 total secs.

Lambda	K	H
0.326	0.142	0.448

Gapped Lambda	K	H
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Matrix: BLOSUM62

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Number of extensions: 11

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effective search space used: 13138503696  
T: 9  
A: 40  
X1: 15 ( 7.0 bits)  
X2: 129 (49.7 bits)  
X3: 129 (49.7 bits)  
S1: 40 (21.6 bits)  
S2: 67 (30.4 bits)